Chapter 3

Quantification of Arginine and Its Methylated Derivatives in Plasma by High-Performance Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

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Abstract

Arginine is the substrate for nitric oxide synthases (NOS), thus the production of nitric oxide (NO) is based on arginine availability. Arginine is methylated through the activity of protein arginine methyltransferases (PRMT1 and PRMT2), to form asymmetrical dimethylarginine (ADMA) and symmetrical dimethylarginine (SDMA). These compounds have gained interest in recent years due to their influence on NO production rates and association with cardiovascular and renal diseases. The accurate and precise measurement of arginine and its methylated derivatives is needed for research studies investigating their role(s) in NO bioavailability and development of disease. We describe a high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method for quantifying arginine, ADMA, and SDMA requiring only 50 μL of plasma. The sample preparation involves addition of internal standards (ADMA-d_7 for ADMA and SDMA, and $^{13}C_6$ -arginine for arginine) prior to protein precipitation with LCMS grade acetonitrile. Samples are centrifuged and supernatant is dried under nitrogen gas at 50 °C. Samples are reconstituted with mobile phase (ammonium acetate—formic acid—water). Arginine, ADMA, and SDMA are separated using an isocratic HPLC method on a 3 μM silica analytical column. MS/MS detection is performed in the multiple-reaction monitoring (MRM) mode and the transitions monitored are m/z 203 to m/z 70 for ADMA and SDMA, m/z 210 to m/z 77 for ADMA-d_7, m/z 175 to m/z 70 for arginine, and m/z 181 to m/z 74 for $^{13}C_6$-arginine.

Key words Arginine, Asymmetric dimethylarginine, Symmetric dimethylarginine, Mass spectrometry, Liquid chromatography, Plasma, Quantification

1 Introduction

Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are the major products of arginine methylation. N-monomethylarginine (NMMA) is an intermediate in this pathway so its concentration is significantly less than that of ADMA and SDMA in plasma. These compounds have gained recent interest due to their role in NO production and evidence that imbalanced NO synthesis leads to loss of vascular "protection", which results in endothelial dysfunction and oxidative stress [1]. Nitric oxide is

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produced from L-arginine in a reaction catalyzed by three distinct isoforms of NO synthase (NOS). NMMA and ADMA directly inhibit NOS, whereas SDMA may limit NO production by competitively inhibiting the cellular uptake of arginine. Recent reports indicate increased concentrations of methylarginine compounds are associated with many pathological conditions, including cardiovascular disease, renal failure, pulmonary hypertension, septic shock, and preeclampsia [2, 3]. The actions of methylated arginines and their contribution to evolution of disease are poorly understood. Investigation into the metabolism of arginine and its methylated derivatives requires accurate and precise measurement of their concentrations in biological samples. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is well suited for this purpose, as it enables simultaneous quantitation of arginine and the methylarginines with high sensitivity, specificity, and precision.

This chapter describes a LC-MS/MS method for quantifying arginine, ADMA, and SDMA in 50 μL of plasma.

2 Materials

2.1 Samples

Plasma collected using K2-EDTA anticoagulant. Samples are stable for 1 month when frozen at −20 °C for up to two freeze/thaw cycles.

2.2 Solvents and Reagents

1. Clinical Laboratory Reagent Water (CLRW) obtained from Millipore Milli-Q Integral 5 Water Purification System.
2. Ammonium Acetate HPLC grade, 1 M, prepared with Special Reagent Water. Stable at 4 °C for 1 month.
3. Mobile Phase A and Purge Solvent (2 mM ammonium acetate/0.1 % (v/v) formic acid in CLRW): Add 2 mL of 1 M ammonium acetate solution and 1 mL formic acid to 1 L water. Stable at room temperature for 2 weeks.
4. Mobile Phase B (2 mM ammonium acetate/0.1 % (v/v) formic acid in methanol): Add 2 mL of 1 M ammonium acetate solution and add 1 mL formic acid to 1 L methanol. Stable at room temperature for 2 weeks.
5. Column Wash Solvent (50 % methanol in water): Mix 500 mL of water and 500 mL of methanol in a 1-L solution bottle. Stable at room temperature for 1 month.
6. Needle Wash Solvent (100 % methanol): Stable at room temperature for 1 month.
7. Charcoal dextran stripped human serum.
8. Phosphate Buffered Saline (PBS), 0.138 M NaCl, 0.0027 M KCl.

2.3 Standards and Calibrators

1. Primary standards: N^G,N^G-Dimethylarginine (ADMA) dihydrochloride ($C_8H_{18}N_4O_2 \cdot 2HCl$), N^G,$N^{G\prime}$-Dimethyl-L-arginine di(*p*-hydroxyazobenzene-*p*′-sulfonate) salt (SDMA) ($C_8H_{18}N_4O_2 \cdot 2C_{12}H_{10}N_2O_4S$), L-arginine ($C_6H_{14}N_4O_2$) (Sigma Aldrich Co.).
2. ADMA Calibrator Stock Solutions (23.3–727 μmol/L primary standard in CLRW):
   (a) Add 10 mg ADMA primary standard to 50-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA standard stock solution 1 is 727 μmol/L. Stable at −70 °C for 2 years (*see* **Note 1**).
   (b) Add 4 mL of the ADMA standard solution 1 to 25-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA standard stock solution 2 is 116 μmol/L. Stable at −70 °C for 2 years.
   (c) Add 2 mL of standard stock solution 2 to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA standard stock solution 3 is 23.3 μmol/L. Stable at −70 °C for 2 years.
3. SDMA Calibrator Stock Solutions (66.2–1380 μmol/L primary standard in CLRW):
   (a) Add 10 mg SDMA primary standard to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This SDMA standard stock solution 1 is 1380 μmol/L. Stable at −70 °C for 2 years (*see* **Note 1**).
   (b) Add 4 mL of the SDMA standard solution 1 to 25-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This SDMA standard stock solution 2 is 221 μmol/L. Stable at −70 °C for 2 years.
   (c) Add 3 mL of standard stock solution 2 to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This SDMA standard stock solution 3 is 66.2 μmol/L. Stable at −70 °C for 2 years.
4. Arginine Calibrator Stock Solution (5473 μmol/L primary standard in CLRW): Add 25 mg arginine primary standard to 25-mL volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at −70 °C for 2 years (*see* **Note 1**).
5. Calibrators (0–2.33 μmol/L ADMA, 0–4.42 μmol/L SDMA, 0–230 μmol/L Arginine in CLRW): Prepare Calibrators 1–5 by diluting the standard stock solution(s) according to Table 1. Working calibrators 1, 2, 3, and 5 are made according to Table 1 using 50-mL volumetric flask(s). Working calibrator 4 is made according to Table 1 using a 100-mL volumetric flask. Working calibrator 5 is CLRW. The calibrators are stable for 2 years when stored at −70 °C (*see* **Note 2**).

Table 1
Preparation of calibrators

Calibrator	Volume of standard stock solution (mL)	Volume of special reagent water (mL)	Final concentration (μmol/L)
1	1.0 (ADMA stock solution 2) 1.0 (SDMA stock solution 2) 2.0 (Arginine stock solution)	46.0	2.33 (ADMA) 4.42 (SDMA) 230 (Arginine)
2	0.5 (ADMA stock solution 2) 0.5 (SDMA stock solution 2) 1.0 (Arginine stock solution)	48.0	1.16 (ADMA) 2.21 (SDMA) 115 (Arginine)
3	1.0 (ADMA stock solution 3) 1.0 (SDMA stock solution 3) 0.5 (Arginine stock solution)	47.5	0.47 (ADMA) 1.32 (SDMA) 57.4 (Arginine)
4	0.5 (ADMA stock solution 3) 0.5 (SDMA stock solution 3) 0.5 (Arginine stock solution)	98.5	0.12 (ADMA) 0.33 (SDMA) 28.7 (Arginine)
5	0	50	0 (ADMA) 0 (SDMA) 0 (Arginine)

6. HPLC/MS Check Stock Solution (34.9 μmol/L ADMA, 11.0 μmol/L SDMA, 2872 μmol/L Arginine in CLRW): Add 3 mL of ADMA standard stock solution 2, 0.5 mL of SDMA standard stock solution 2, and 5 mL of arginine standard stock solution to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at −70 °C for 2 years.
7. HPLC/MS Check Standard (0.55 μmol/L ADMA, 0.11 μmol/L SDMA, 28.7 μmol/L Arginine in CLRW): Add 0.5 mL of HPLC/MS Check stock solution to 50-mL volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at −70 °C for 2 years.

2.4 Internal Standard and Quality Controls

1. Primary internal standards (I.S.): asymmetric dimethylarginine hydrochloride (ADMA-d_7) ($C_8H_{12}ClD_7N_4O_2 \cdot H_2O$), $^{13}C_6$-L-arginine hydrochloride ($^{13}C_6H_{15}ClN_4O_2$) (Cambridge Isotope Laboratories).
2. Quality Control Stock solutions: Primary standards are separately weighed or from different lots than those used to prepare calibrator stock solutions.
3. ADMA-d_7 I.S. Stock Solution (379 μmol/L primary I.S. in CLRW): Add 5 mg ADMA-d_7 primary I.S. to 50-mL volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at −70 °C for 2 years (*see* **Note 1**).
4. $^{13}C_6$-Arginine I.S. Stock Solution (923 μmol/L primary I.S. in CLRW): Add 10 mg $^{13}C_6$-arginine primary I.S. to 50-mL

volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at –70 °C for 2 years (*see* **Note 1**).

5. I.S. Working Solution (1.90 μmol/L ADMA-d_7, 36.9 μmol/L $^{13}C_6$-Arginine in CLRW): Add 0.5 mL of ADMA-d_7 I.S. stock solution and 4 mL of $^{13}C_6$-Arginine I.S. stock solution to 100-mL volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at –70 °C for 2 years (*see* **Note 3**).
6. ADMA Quality Control Stock Solutions (7.27–3635 μmol/L primary standard in CLRW):
   (a) Add 10 mg ADMA primary standard to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA quality control stock solution 1 is 3635 μmol/L. Stable at –70 °C for 2 years (*see* **Note 1**).
   (b) Add 5 mL of previous ADMA quality control stock solution 1 to 50-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA quality control stock solution 2 is 364 μmol/L. Stable at –70 °C for 2 years.
   (c) Add 2 mL of ADMA quality control stock solution 2 to 100-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA quality control stock solution 3 is 7.27 μmol/L. Stable at –70 °C for 2 years.
7. SDMA Quality Control Stock Solutions (2.76–138 μmol/L primary standard in CLRW):
   (a) Add 10 mg SDMA primary standard to 100-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This SDMA quality control stock solution 1 is 138 μmol/L. Stable at –70 °C for 2 years (*see* **Note 1**).
   (b) Add 2 mL of previous SDMA quality control stock solution 1 to 100-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This SDMA quality control stock solution 2 is 2.27 μmol/L. Stable at –70 °C for 2 years.
8. Arginine Quality Control Stock Solutions (2297–4594 μmol/L primary standard in CLRW).
   (a) Add 20 mg of arginine primary standard to 25 mL volumetric flask, bring to volume with CLRW and mix well by inversion. This arginine quality control stock solution 1 is 4594 μmol/L. Stable at –70 °C for 2 years (*see* **Note 1**).
   (b) Add 5 mL of the previous arginine quality control stock solution 1 to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This arginine quality control stock solution 2 is 2297 μg/mL. Stable at –70 °C for 2 years.

Table 2
Preparation of quality controls

Quality control	Volume of control stock solution (mL)	Volume of charcoal dextran stripped serum (mL)	Final concentration (μmol/L)
Low	1.0 (ADMA stock solution 3) 3.0 (SDMA stock solution 2) 0.5 (Arginine stock solution 2)	20.5	0.29 (ADMA) 0.33 (SDMA) 66.4 (Arginine)
High	2.0 (ADMA stock solution 3) 5.0 (SDMA stock solution 2) 2.0 (Arginine stock solution 2)	16.0	0.58 (ADMA) 0.55 (SDMA) 200 (Arginine)

9. Quality Controls (0.29–0.58 μmol/L ADMA, 0.33–0.55 μmol/L SDMA, 45.9–184 μmol/L, Arginine in charcoal stripped serum).
   (a) The levels of ADMA, SDMA, and arginine in the serum or plasma are predetermined by using this protocol or by an external laboratory using LC-MS/MS technology and are added to the final concentrations of the quality controls.
   (b) Prepare low and high controls by diluting quality control stock solutions as shown in Table 2. For each dilution step: Add appropriate amount of quality control stock solution(s) for each ADMA, SDMA, and arginine as shown in Table 2 to 25-mL volumetric flask(s) and bring to volume with serum (or plasma). Mix well by inversion after each dilution step. Stable at −70 °C for 2 years (*see* **Notes 2** and **4**).

2.5 Supplies and Equipment

1. Analytical Column: Phenomenex Luna Silica, 3 μm 100 × 4.6 mm I.D.
2. Guard Column: Phenomenex Luna Silica, 4 × 3.0 mm I.D.
3. Waters 2795 Alliance HT Separation Module with Micromass Quattro Micro API equipped with MassLynx.
4. Thermo Scientific Reacti-Therm III Heating/Stirring Module.

3 Methods

3.1 Stepwise Procedure

1. Ensure the instrument is properly tuned and verify system performance (*see* **Notes 5** and **6**).
2. Pipette 50 μL of sample (calibrators, quality controls, PBS blank, and patient plasma) to labeled 1.5-mL microcentrifuge tubes.
3. Add 50 μL of the internal standard solution.
4. Cap and vortex mix tubes briefly and let stand for 10 min at room temperature.
5. Add 500 μL of acetonitrile to each tube, cap and vortex mix for 7–10 s.

6. Let the samples stand for 15 min at room temperature.
7. Centrifuge at 13,440 rcf for 5 min at room temperature.
8. Transfer supernatant into labeled 13 × 100 mm glass culture tubes.
9. Using the Thermo Scientific Reacti-Therm III Heating/Stirring Module, dry samples gently under nitrogen gas at 50 °C until completely dry (*see* **Note 7**).
10. Reconstitute the supernatant by adding 1.0 mL mobile phase A.
11. Cap the tubes and vortex mix thoroughly for 7–10 s.
12. Centrifuge at 1430–1500 × *g* for 5 min.
13. Transfer solution to appropriately labeled autosampler vials.
14. Inject 10 μL of sample onto LC-MS/MS.

3.2 Analysis

1. Instrumental operating parameters are given in Table 3.
2. Analyze the data using the QuanLynx software (Waters Corporation).

Table 3
LC-MS/MS operating conditions

A. HPLC[a]			
Column temperature		10 °C ±5	
Flow rate		0.375 mL/min	
Isocratic program		Time (min)	
		0.00–9.00	
		95 % Mobile Phase A	
B. MS/MS tune settings[b]			
Capillary voltage (kV)		0.25	
Source temperature (°C)		130	
Desolvation temperature (°C)		450	
Cone gas (L/h)		30	
Desolvation gas (L/h)		650	
Collision gas pressure (mbar)		4.17 e–3	
LM1 resolution		13.5	
HM1 resolution		13.5	
Ion energy 1		0.1	
MS/MS entrance		–2	
MS/MS exit		2	
LM2 resolution		11.3	
HM2 resolution		11.3	
Ion energy 2		2.4	
C. MRM method settings[b]			
Cone (V)	Collision (eV)	Inter-channel delay (s)	Inter-scan delay (s)
27	23	0.03	0.03

[a]The total run time is 9.0 min. Solvent flow was diverted from the source to waste at 0–2 min and at 8.5–9.0 min
[b]Tune and MRM settings may vary slightly between instruments

Table 4
Precursor and product ions for ADMA, SDMA, ADMA-d_7, arginine, and $^{13}C_6$-arginine

Analyte	Precursor ion $(M+H)^+$	Product ion
ADMA	203.10	69.95
SDMA	203.10	69.95
ADMA-d_7	210.15	76.95
Arginine	175.10	69.90
$^{13}C_6$-Arginine	181.10	73.95

3. With each analytical run, a 5-point standard calibration curve is created by linear regression of the analyte/I.S. peak area ratio with the origin included using the quantifying ions indicated in Table 4. The concentrations of the controls and unknown samples are determined from the curve.
4. The expected retention times for ADMA, SDMA, and arginine are 6.77 min (acceptable range: 6.43–7.11 min), 6.04 min (acceptable range: 5.74–6.32 min), and 4.27 min (acceptable range: 4.20–4.45 min), respectively. The expected retention times for ADMA-d_7 and $^{13}C_6$-Arginine are 6.77 min (acceptable range: 6.43–7.11 min) and 4.26 min (acceptable range: 4.20–4.45 min), respectively. Representative ion chromatograms for ADMA, SDMA, arginine, and I.S. are shown in Fig. 1.
5. Verify the performance during the analytical run by monitoring the internal standard peak area. An acceptable limit should be defined during method development or validation. We determined 1500 to be the minimum acceptable IS peak area in our method. Re-inject the sample if the internal standard peak area is below the acceptance limit of 1500. If after re-injection, the internal standard peak area is still below 1500, determine the signal-to-noise ratio of the analyte peak. Signal-to-noise ratio greater than 10 is acceptable for reporting.
6. Evaluate for carryover effects in the PBS blank injected after Calibrator 1. Carryover is significant when ADMA, SDMA, and arginine concentrations in the PBS blank is greater than the limit of detection levels 0.01, 0.03, and 1.15 μmol/L, respectively, and in the low quality control is greater than the two standard deviations of the target value and/or assigned mean. If carryover is significant, troubleshoot and perform corrective action. Repeat the evaluation to demonstrate that carryover is no longer detected.
7. Run is acceptable if the calculated concentrations in the control samples are within two standard deviations of the target values and/or assigned means.

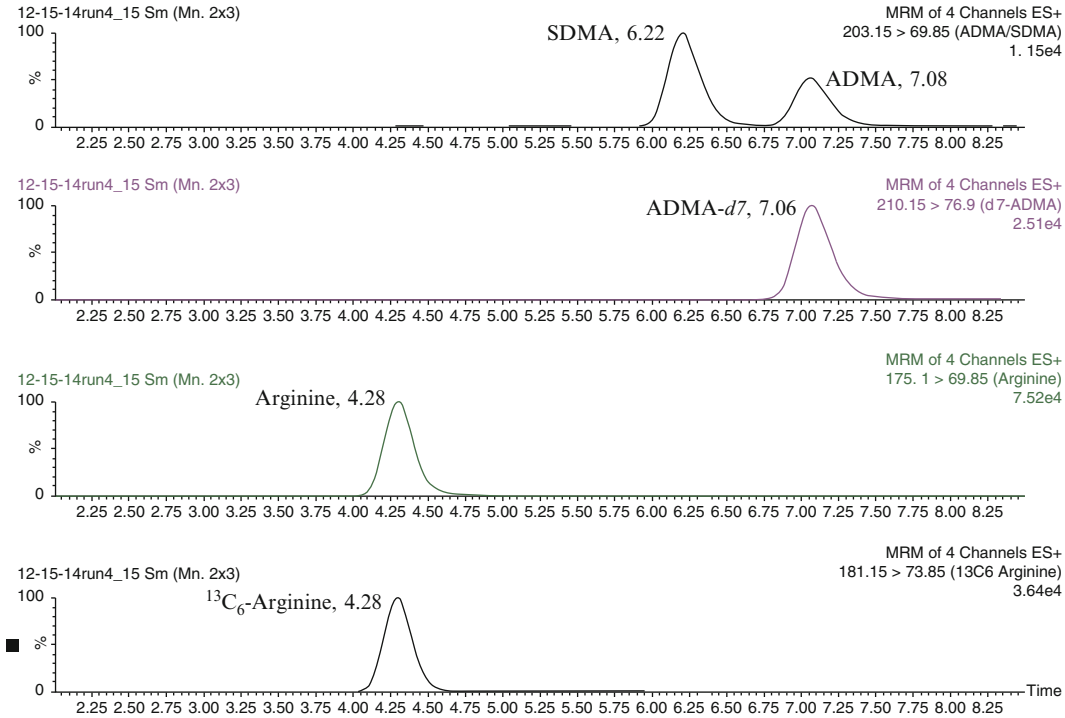

Fig. 1 Representative LC-MS/MS ion chromatograms of SDMA (0.71 μmol/L), ADMA (0.69 μmol/L), ADMA-d_7 (1.9 μmol/L), arginine (62.5 μmol/L), and $^{13}C_6$-arginine (36.1 μmol/L) in a plasma sample

8. The method is linear from 0.10 to 2.15 μmol/L for ADMA, 0.10–6.00 μmol/L for SDMA, and 5.8–230.0 μmol/L for arginine. The low limit of quantitation of ADMA, SDMA, and arginine is 0.10, 0.10, and 5.8 μmol/L, respectively. The intra- and inter-day precision is <5 %.
9. No significant ion suppression was found in charcoal-stripped serum (used for preparing quality controls) and plasma pools.

4 Notes

1. When preparing standard and I.S. stock solution(s), completely dissolve the solid material in small amount of CLRW in volumetric flask(s) before bringing to volume. Briefly sonicate to accelerate the dissolution process.
2. Calibrators and quality controls are pre-aliquoted and stored in −70 °C until use. Pipette 125 μL of the calibrator and quality controls solutions into 1.2-mL cryogenic vials. Opened calibrator vials are for one time use only. Opened quality control vials are stable for 7 days at −20 °C.
3. Working I.S. solution is pre-aliquoted and stored in −70 °C until use. Pipette 2 mL of the solution into 2.0-mL cryogenic vials.

Opened vials are for one time use only. When more than one vial of the solution is required to prepare a batch of samples, combine and mix well before use.

4. Charcoal dextran stripped serum is preferred for preparing quality controls because it contains minimal level of endogenous ADMA, SDMA, and arginine. However, pooled or single-donor serum and/or EDTA plasma obtained from healthy volunteers may also be used. The serum or plasma can be diluted with PBS to reduce the concentration of endogenous ADMA, SDMA, and arginine. The percentage of PBS compared to serum or plasma should not exceed 50 %.
5. System check: To verify system performance before running patient samples, inject the HPLC/MS check standard solution after a water blank. Verify that the analytes retention times are within their respective acceptable limits and that the signal-to-noise (peak-to-peak) of the ADMA and arginine peaks is greater than 100 and greater than 10 for the SDMA peak. The HPLC/MS check standard solution is pre-aliquoted and stored in −70 °C until use. Opened vials are for one time use only. New columns are prepared by flushing with 10 mL of 100 % isopropanol alcohol at 0.400 mL/min followed by 50 mL of 100 % methanol at 0.400 mL/min.
6. Tuning the mass spectrometer: To adjust the mass spectrometer parameters for optimum sensitivity and stability of ions measured, tuning solutions of ADMA, SDMA, ADMA-d_7, arginine, and $^{13}C_6$-arginine (12, 40, 10, 4, and 4 μg/mL in CLRW, respectively) are infused into the ion source at 10 μL/min while solvent from the HPLC consisting of 95 % Mobile Phase A and 5 % Mobile Phase B is introduced via a peak "tee" connector at 0.375 mL/min. After analytical runs are completed, the column is flushed for 60 min at a flow rate of 0.200 mL/min and stored with 70 % methanol in water.
7. Apply low nitrogen gas flow during the drying step. Analyte may be lost at higher gas flow rates.

References

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